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TSC Patient-Derived Isogenic Neural Progenitor Cells Reveal Altered Early Neurodevelopmental Phenotypes and Rapamycin-Induced MNK-eIF4E Signaling

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Background: Tuberous sclerosis complex (TSC) is a neurodevelopmental disorder with frequent occurrence of epilepsy, autism spectrum disorder (ASD), intellectual disability (ID), and tumors in multiple organs. The aberrant activation of mTORC1 in TSC has led to treatment with mTORC1 inhibitor rapamycin as a lifelong therapy for tumors, but TSC-associated neurocognitive manifestations remain unaffected by rapamycin. Methods: Here, we generated patient-specific, induced pluripotent stem cells (iPSCs) from a patient with TSC with a heterozygous, germline, nonsense mutation in exon 15 of TSC1 and established an isogenic set of heterozygous (Het), null, and corrected wild-type (Corr-WT) iPSCs using CRISPR/Cas9-mediated gene editing. We differentiated these iPSCs into neural progenitor cells (NPCs) and examined neurodevelopmental phenotypes, signaling, and changes in gene expression by RNA-seq. Results: Differentiated NPCs revealed enlarged cell size in TSC1-Het and Null NPCs, consistent with mTORC1 activation. TSC1-Het and Null NPCs also revealed enhanced proliferation and altered neurite outgrowth in a genotype-dependent manner, which was not reversed by rapamycin. Transcriptome analyses of TSC1-NPCs revealed differentially expressed genes that display a genotype-dependent linear response, that is, genes upregulated/downregulated in Het were further increased/decreased in Null. In particular, genes linked to ASD, epilepsy, and ID were significantly upregulated or downregulated, warranting further investigation. In TSC1-Het and Null NPCs, we also observed basal activation of ERK1/2, which was further activated upon rapamycin treatment. Rapamycin also increased MNK1/2-eIF4E signaling in TSC1-deficient NPCs. Conclusion: MEK-ERK and MNK-eIF4E pathways regulate protein translation, and our results suggest that aberrant translation distinct in TSC1/2-deficient NPCs could play a role in neurodevelopmental defects. Our data showing upregulation of these signaling pathways by rapamycin support a strategy to combine an MEK or an MNK inhibitor with rapamycin that may be superior for TSC-associated central nervous system defects. Importantly, our generation of isogenic sets of NPCs from patients with TSC provides a valuable platform for translatome and large-scale drug screening studies. Overall, our studies further support the notion that early developmental events such as NPC proliferation and initial process formation, such as neurite number and length that occur prior to neuronal differentiation, represent primary events in neurogenesis critical to disease pathogenesis of neurodevelopmental disorders such as ASD.

Commentary

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disease with an estimated incidence of 1:6000 to 1:10 000 live birth caused by pathogenic variants in *TSC1* (hamartin) or *TSC2* (tuberin) genes that together with *TBC1D7* act as the main negative regulator of the mechanistic target of rapamycin (mTOR) signaling pathway. Mechanistic target of rapamycin further associates in 2 protein complexes, mTORC1 and mTORC2, that manifest distinct roles.[1] In the brain, mTORC1 regulates protein and lipid synthesis, cell growth, metabolism, and autophagy and has established functions in neuronal excitability, memory formation, and learning. mTORC2 is primarily involved in the maintenance of cytoskeletal integrity and cell migration.[1,2] Hyperactivation of the mTOR signaling pathway subsequent to loss-of-function variants in either *TSC1* or *TSC2* results in abnormal cellular morphology, proliferation, and multi-organ hamartomatosis.[1] Sirolimus (rapamycin) and everolimus (Afinitor) are macrolide derivatives that were identified as potent mTORC1 inhibitors, and their clinical use demonstrated efficacy for the treatment of renal angiomyolipoma, subependymal giant cell astrocytoma (SEGA), and lymphangioleiomyomatosis.[1] A mouse model of TSC with conditional inactivation of the *Tsc1* gene in glial fibrillary acidic protein (GFAP)-positive cells ($Tsc1^{GFAP}$CKO

mice) manifested time-dependent control of epilepsy when treated with sirolimus. Early treatment prior to the development of clinical seizures at postnatal day 14 suppressed the onset of experimental epilepsy for the duration of treatment, while treatment after spontaneous seizures were well established and resulted in an improvement but not a full control of seizures.[3] Clinical trials paralleled the preclinical experience, and treatment of patients with TSC-related medically refractory epilepsy with everolimus resulted in a significant reduction in seizure frequency, although most patients with TSC did not become seizure-free.[4-6] Furthermore, epilepsy-related burden is only one of several challenging consequences of TSC, as patients typically also manifest autism spectrum disorder and a variable degree of intellectual disability.[1,2] While modulating hyperactive mTOR pathway with everolimus may lead to a meaningful improvement in epilepsy, its effect on cognition and behavior remains less well defined. Although preclinical data suggested improvement, a 6-month long administration of everolimus to children with TSC in a randomized, placebo-controlled trial showed a possible trend but not a statistically significant improvement in neurocognitive functioning or in behavior, and similar results were noted in EXIST-3 substudy in Japan.[5,7] Considering the logistical challenges of these trials (patient heterogeneity, confounding effects of medications, comorbid conditions, and others), results of these clinical studies hardly mean absolute lack of efficacy but rather highlight the biological complexity that underlies neurocognitive dysfunction in TSC. As illustrated in the $Tsc1^{GFAP}CKO$ model, sirolimus administration before the clinical onset of seizures ameliorated progressive astrogliosis and abnormal neuronal organization, and it suppressed the development of interictal and ictal abnormalities.[3] Treatment timing may similarly be critical when aiming to salvage cognition. Furthermore, individual genetic background may influence the neurological phenotype and treatment response, and this variable is difficult to model in a mouse. The application of patient-specific induced pluripotent stem cells (iPSCs) is transcending some limits inherent to genetic mouse models since iPSCs preserve patients' genetic background and are amenable to molecular research and drug testing.

Martin et al took advantage of the unique attributes of the iPSC model system as they aimed to contribute toward a deeper understanding of neurocognitive consequences observed in TSC. They explored molecular effects of a private *TSC1* gene pathogenic variant and the effects and limitations of sirolimus (rapamycin) in patient-specific skin fibroblast-derived iPSCs.[8] The patient affected by TSC carried a truncating nonsense variant in *TSC1* exon 15 (1746C>T, Arg509X). Using CRISPR/Cas9 technique, the investigators generated a null *TSC1*-iPSCs as well as "corrected" wild-type (Corr-WT) of the heterozygous *TSC1*-Het iPSCs with *TSC1* (1746C>T, Arg509X) variant. The isogenic iPSCs (Het, Null, and Corr-WT) were then differentiated into neural progenitor cells (NPCs). As would be expected, compared to Corr-WT, the TSC1-Het and Null NPCs were larger in size and they proliferated faster in a dose-dependent manner, a finding consistent with the activation of mTORC1 complex reflected in downstream increase in expression levels of the phosphoribosomal protein S6. The TSC-Het and Null also showed increased number and length of neurites. Signaling through mTORC1 and mTORC2 complexes was shown to be important for the development and morphology of dendrites and axonal outgrowth.[2] Administration of rapamycin reduced cell size but not the proliferation rate, and it did not affect neurite length or number. This finding is supported by prior experimental studies in a *Tsc1* knockout mice, which has shown that dendritic patterning is modulated in an mTOR-independent manner through mitogen-activated protein kinases (MEK) that regulate phosphorylation of extracellular signal-regulated kinases (ERK).[2,9] The MEK-ERK was shown to be aberrantly activated in SEGAs,[8] and a similar activation and elevation of phosphorylated ERK (pERK) was noticed in the current study. Interestingly, pretreatment with rapamycin, while blocking mTORC1 activation, led to a significant increase in pERK1/2 in TSC-Het and Null and it was abolished by an application of MEK inhibitor trametinib. An MEK-related pathway and interaction important for the dynamic process of neuronal synaptic plasticity is mitogen-activated protein kinase–interacting kinase (MNK). The MNK phosphorylates and thus regulates the binding of eukaryotic translation initiation factor 4E (eIF4E) to its target proteins, such as fragile X mental retardation protein (FMRP) and cytoplasmic fragile X protein-interacting protein 1 (CYFIP1). The FMRP/CYFIP1 complex then regulates FMRP targets and messenger RNAs involved in cytoskeletal regulation and growth.[10] In the study by Martin et al, the baseline phosphorylation status of eIE4A was comparable among Corr-WT, TSC-Het, and Null, but rapamycin application led to PI3K-dependent activation of MNK-eIE4A signaling. These findings, at least in part, may explain the sustained cellular proliferation and neurite length and number despite rapamycin treatment. Transcriptome comparative profiling of the TSC-Het and Null versus Corr-WT uncovered 107 differentially expressed genes shared between TSC-Het and Null, and 29 of 107 genes showed gene dose-dependent up- or downregulation. Among the dysregulated genes were the zinc-finger family of DNA-binding transcription factors, transcriptional regulators, and notably *PCDH19*, *PCDH10*, *ANXA1*, *CNTN6*, and *HLA-B* with a known association with epilepsy, ASD, and ID.

To date, most human neuronal models have evaluated molecular consequences of *TSC2* defects,[11] while the study by Martin et al assessed dose-dependent effects and consequences of a mutant *TSC1* gene, an important complement to the research on cellular phenotypic impact of pathogenic variants in the 2 main *TSC* genes. Results contribute to an emerging body of literature, indicating that early abnormalities in the proliferation of NPCs, neurite outgrowth, and migration likely contribute to a subsequent development of neurocognitive dysfunction later in life. This work also shows that *TSC1* loss-related early neurodevelopmental phenotypes do not exclusively depend on mTORC1 activation. This suggests that activation of an alternative signaling pathway mediated by MEK-ERK and MNK-

eIF4A mechanisms may be contributory to the cognitive and neuropsychiatric consequences of TSC. While the authors demonstrate effective selective inhibition of MEK-ERK and MNK-eIF4A pathways in NPCs on protein levels, it remains unclear whether this translates into a normalized cellular phenotype. Similarly, the study stops short of testing the effect of a combined inhibition of mTORC1 and MEK-ERK and MNK-eIF4A pathways on the NPC phenotype. Furthermore, there is an absence of cellular electrophysiology to relate the findings of perturbed transcriptome and molecular and cellular phenotypes to epilepsy. Aside from some of these immediate questions, this study makes one wonder about processes and adaptive changes that occur either as early sequelae of *TSC1* gene deficiency or following treatment with rapamycin analogs at different stages of neural and disease development. It also highlights candidate therapeutic targets that will need further exploration in search for treatment and prevention of epilepsy and cognitive consequences of TCS.

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